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A canine model of irreversible urethral sphincter insufficiency

Eberli, D ; Andersson, K E ; Yoo, J J ; Atala, A

Abstract: **OBJECTIVE** To develop a canine model of external urinary sphincter insufficiency by creating irreversible damage to the sphincter, because there is a need for a reliable and reproducible large animal model for the study of stress urinary incontinence (SUI) caused by deficient sphincter function. **MATERIALS AND METHODS** About a quarter of the total external sphincter muscle was removed microscurgically from seven female dogs; three age-matched dogs served as normal controls. The dogs had standard urodynamic and radiographic studies before and at 1, 2, 3, 4 and 7 months after surgery. Three dogs were killed at 4 months and four at 7 months after surgery for tissue analyses. **RESULTS** The interventions produced a consistent outcome. Urodynamic studies showed a significant and sustained decrease in sphincter function, which included a static urethral pressure profile, stress urethral profile and detrusor leak-point pressure. Furthermore, in vivo pudendal nerve stimulation and organ-bath studies of the retrieved tissue strips confirmed the loss of sphincter tissue function. Histologically, absence of functional sphincter muscle was evident in the damaged sphincter region. **CONCLUSIONS** These results show that a reliable and reproducible canine model of irreversible sphincter insufficiency can be created by microsurgical removal of sphincter muscle tissue. This model of external sphincter insufficiency could be used for evaluating methods (e.g. cell therapies) for treating SUI.

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A Canine Model of Irreversible Urethral Sphincter Insufficiency

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ABSTRACT

Objective: To develop a canine model of external urinary sphincter insufficiency by creating irreversible damage to the sphincter, since there is a need for a reliable and reproducible large animal model for study of stress urinary incontinence (SUI) caused by deficient sphincter function.

Materials and Methods: Approximately 25% of the total external sphincter muscle was removed microscurgically from 7 female dogs; 3 age matched animals served as normal controls. Standard urodynamic and radiographic studies were performed before and at 1, 2, 3, 4 and 7 months after surgery. Three animals were sacrificed at 4 months and 4 at 7 months after surgery for tissue analyses.

Results: The interventions produced a consistent outcome. Urodynamic studies showed a significant and sustained decrease in sphincter function, which included static urethral pressure profile, stress urethral profile and detrusor leak point pressure. Furthermore, *in vivo* pudendal nerve stimulation and organ bath studies of the retrieved tissue strips confirmed the loss of sphincter tissue function. Histologically, absence of functional sphincter muscle was evident in the damaged sphincter region.

Conclusions: These results show that a reliable and reproducible canine model of irreversible sphincter insufficiency can be achieved through microsurgical removal of sphincter muscle tissue. This model of external sphincter insufficiency may be used for evaluation of e.g., cell therapies for the treatment of SUI.

KEY WORDS: animal disease model; urodynamic studies; stress urinary incontinence.

Introduction

Irreversible damage to the external urinary sphincter is a main cause of involuntary leakage of urine [1]. Sphincter damage may be caused by various conditions including congenital anomaly, trauma, surgery and vaginal child birth, which lead to stress urinary incontinence (SUI) [2]. Current treatments of SUI include pharmacological therapy [3], bulking agents [4], novel surgical approaches [5], and cellular therapy [6-8]. All these modalities have limitations, and many innovative approaches have been proposed experimentally to improve treatment success. To develop new therapies for SUI caused by sphincter insufficiency, a reliable animal model that mimics the human clinical condition is needed. Several rodent models have been used to demonstrate the feasibility of new therapies. However, they often lack reliability due to inaccurate functional assessment. In addition, these animal models do not recapitulate the conditions seen in patients. Nonetheless, rodent models have been a valuable tool in the development of various therapies [9, 10]. The limitations of current therapies have recently focused attention to stem cells and tissue engineering for treatment of SUI [6-8]. Tissue engineering involves the use of cell transplantation and materials science to develop a biological substitute for therapeutic applications. Several studies have suggested that muscle progenitor cells (MPCs) injected into the rhabdosphincter survive and differentiate into regenerative myofibers [9-12]. To make possible extended studies of this therapeutic approach, well characterized large animal models are desirable. A useful model replicating irreversible loss of external sphincter function, should fulfill several criteria: 1) damage should be sustained to allow for long-term studies, 2) sphincter function should be measurable using established tools such as urodynamic studies, and 3) the model should be easily created and maintained. A canine model may fulfill these criteria. This species has been previously used for lower urinary tract research, and it is morphologically considered to be a suitable model for investigations of the urethral sphincter [13]. In addition, urodynamic evaluations in these species have been reported [14-16]. Therefore, we developed and characterized a canine model of SUI based on irreversible damage to the external urethral sphincter.

Material and Methods

Study design

The study was performed on 10 adult female beagles weighing 10 to 12 kg. All animals were 4 to 5 years of age. External urethral sphincter muscle damage was surgically created in 7 animals, 4 of which were followed for up to 7 months. Three age-matched normal animals served as controls. Routine urodynamic evaluation was performed before surgery and every month thereafter until a total of 42 urodynamic studies were performed. Radiographic studies of the bladder neck were performed followed by gross examination, physiological organ bath studies and histological analyses of the sphincter tissue.

Anatomy and Surgical technique

The anatomy of the smooth and striated muscles of the female canine urethra has been described in detail by [13, 17]. The sphincter muscle, which has no muscular connection to the pelvic floor, has smooth and striated muscular elements. The striated component is located in the lower cranial third of the membranous urethra. It covers the ventral aspect of the smooth muscle, and completely encircles the smooth muscle in the upper middle third. Distally, the striated part of the urethral sphincter is located around the urethra and the vagina. The circular orientation of the sphincter muscle (*musculus urethralis*) in dogs is equivalent to the external urogenital sphincter in humans, which is composed of compressor urethrae and the urethrovaginal sphincter. In dogs, the external sphincter is thus composed of skeletal muscle from the *musculus urethralis* (pelvic floor equivalent) and smooth muscle from the muscle layer of the urethra [13, 17]. Despite the anatomical differences between canines and human, the sphincter muscle functions similarly. At the site of maximal closure pressure its two muscle components combine and build one entity - the external sphincter. Due to the importance of the external sphincter for continence, this study focused solely on the external sphincter as one functional unit.

All experimental procedures were reviewed and approved by the Wake Forest University Institutional Animal Care and Use Committee, and were performed in compliance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. Anesthesia was induced with 12 mg/kg Telazol® (tiletamine and zolazepam) intramuscularly (Fort Dodge, Fort Dodge, Iowa, USA) and maintained with 1% to 2% isoflurane.

A 2 cm longitudinal incision was made on the anterior urethra through a low midline abdominal incision, and the circumferential sphincter muscle was microsurgically removed (Figure 1). The excision of 2 cm sphincter muscle removed approximately 25% of the total sphincter muscle. The proximal and distal margins of the defect were marked with 3-zero nonabsorbable polypropylene sutures followed by abdominal wall closure.

Urodynamic evaluation

The depth of anesthesia was carefully monitored for urodynamic evaluation. A micro-tip transducer catheter (Millar Instruments Inc., Houston, Texas, USA) was inserted into the bladder transurethrally and residual urine was evacuated. Rectal pressure was measured with a balloon catheter attached to a transducer (Life-Tech Inc., Stafford, Texas, USA). Detrusor leak point pressure (DLP) was measured by filling the bladder at a rate of 14 ml per minute and pressure at first leakage was recorded (Life-Tech Urolab Opus System V, Life-Tech Inc., Houston, Texas, USA). Urethral pressure profilometry (UPP) and urethral stress profile (USP) was recorded by withdrawing the sensor catheter at a rate of 0.5 mm per second. During the USP abdominal pressure was applied by hand every 3 to 5 seconds (Crede's maneuver) to mimic coughing. Sphincter closure pressure was calculated by subtracting the measured pressures from the bladder pressure. The average of 3 measurements in the region of the external sphincter was used for quantitative analysis.

Pudendal nerve stimulation

Active external sphincter contraction was assessed by placing the sensor catheter transurethrally in the sphincter region and selectively stimulating the pudendal nerve through a median laparotomy with a setting of 1 ms delay and 1 ms pulse duration at 8 Hz, 24 V (Grass S48 stimulator, Grass Technologies, West Warwick, Rhode Island, USA) [18]. All assessments were repeated and measurements were averaged.

Radiographic studies

Cystourethrography was performed in all animals at the time of sacrifice by infusing contrast media into the bladder until a pressure of 40 cm H₂O was achieved. Anteroposterior images of the lower urinary tract were taken (Siremobil Compact L, Siemens Medical Solutions USA, Malvern, Pennsylvania, USA), and the entire sphincter region was assessed.

Tissue retrieval and gross examination.

A midline incision was made and the symphysis pubis was removed to expose the entire sphincter region. The sphincter area was carefully inspected for signs of inflammation and fibrosis, followed by en bloc removal of the bladder and urethra. The sphincter area, which was marked by nonabsorbable suture material, was examined and cut into 6 equal segments for organ bath and histological studies.

Histology

The retrieved tissue samples were placed in Tissue-Tek® O.C.T. Compound 4583 (Sakura®), frozen in liquid nitrogen and sectioned into 6 µm thin slices using a cryostat (Model CM 1850, Leica Microsystems, Bannockburn, Illinois, USA). The sections were fixed and stained with hematoxylin and eosin. Sphincter muscle cells were confirmed with anti-desmin, which stains positive for skeletal and smooth muscle (1:50, BD Biosciences, San Jose, California, USA). Immunolabeling was performed using the avidin-biotin detection kit (Vectastain Elite ABC, Vector Laboratories Inc, Burlingame, California, USA). All sections were counterstained with Gill's hematoxylin. Native tissue sections served as a positive control and tissue sections without primary antibodies served as the negative controls. Digital images (Zeiss Axio Imager M1 Microscope, Carl Zeiss, Thornwood, New York, USA) were captured at varying magnifications for further analyses.

Organ bath studies

The tissue specimens of the external sphincter were placed in Krebs' solution (NaCl 119 mM, KCl 4.4 mM, NaHCO₃ 20 mM, NaH₂PO₄ 1.2 mM, MgCl₂ 1.2 mM, CaCl₂ 2.5 mM and glucose 11 mM in distilled water, pH 7.2). Muscle strips, 3 from each animal, measuring 2 x 3 x 1 mm, were suspended to measure the circular component of sphincter contraction in a Radnoti tissue bath chamber (Radnoti, Monrovia,

California) with Krebs' solution aerated with 95% O₂ and 5% CO₂ at 37C, and the samples were allowed to equilibrate for 10 minutes under tension of 1.5 g. The muscle strips were stimulated by electrical field stimulation using a Grass S48 stimulator (Grass Technologies, West Warwick, Rhode Island, USA) with the setting of 2 ms delay and 2 ms pulse duration at 100 Hz, 100 V for 1.5 seconds. Maximum tension under tetanic contractions were recorded and normalized to the sample weight (mg/mg tissue).

Statistics

All data were expressed as averages with standard deviations, and were analyzed by paired or unpaired t tests using statistical software (SPSS v11; SPSS Inc., Chicago, Illinois, USA). A p value of less than 0.05 was considered significant.

Results

All animals recovered from surgery without any complications and there was no evidence of bladder infection, inflammation or stone formation during the study. Tissue retrieval was uneventful. No inflammation, infection or fibrosis was observed at the site of sphincter damage.

Urodynamic evaluation

Sphincter function was assessed before and at 1, 2, 3, 4 and 7 months after the model generation (7 dogs at 0 to 4 months and the remaining 4 dogs at 7 months). None of the animals showed signs of detrusor overactivity. The static UPP demonstrated a significant decrease in sphincter pressures after muscle damage from 15.6 ± 4.3 to 3.7 ± 2.6 cm H₂O ($p=0.007$) at 1 month and remained low throughout the entire study period (Figure 2). The average pressure values were 2.1 ± 1.6 cm H₂O ($p=0.001$) at 2 months, 2.6 ± 0.9 cm H₂O ($p < 0.001$) at 3 months, 3.2 ± 1.9 cm H₂O ($p < 0.001$) at 4 months and 2.7 ± 1.4 cm H₂O ($p=0.007$) at 7 months. The USP showed similar results with a steep and significant decrease after sphincter damage from 23.4 ± 9.2 to 5.7 ± 5.2 cm H₂O ($p=0.043$) at 1 month and remained low throughout the study period. The pressure values were 2.75 ± 3.8 cm H₂O ($p=0.004$) at 2 months, 3.5 ± 2.9 cm H₂O ($p=0.002$) at 3 months, 4.0 ± 3.3 cm H₂O ($p=0.002$) at 4 months and 4.7 ± 2.2 cm H₂O ($p=0.045$) at 7 months. Sphincter function did not recover in any of the animals.

DLP measured at maximum bladder capacity showed a significant decrease after damage from 53.0 ± 12.9 to 30.5 ± 13.5 cm H₂O ($p=0.013$) at 1 month and remained significantly low throughout the study period. The pressure values were 19.3 ± 8.8 cm H₂O ($p < 0.001$) at 2 months, 36.0 ± 14.6 cm H₂O ($p=0.016$) at 3 months, 34.5 ± 18.1 cm H₂O ($p=0.001$) at 4 months and 32.5 ± 18.5 cm H₂O ($p=0.018$) at 7 months. Although the DLP remained significantly lower than normal, there was a slight increase in pressure after 3 months.

Pudendal nerve stimulation

Pudendal nerve stimulation triggered sphincter muscle contraction with associated pressure increase. However, the contractile response was significantly lower after sphincter muscle damage (Figure 2C). The pressure response due to stimulation was 20.9 ± 8.7 cm H₂O in normal animals, which decreased to

6.3 ± 2.4 cm H₂O ($p=0.039$) at 4 months and 5.4 ± 1.0 cm H₂O ($p=0.012$) at 7 months in animals whose muscle was removed.

Organ bath studies

Sphincter muscle strips harvested at 4 and 7 months after damage showed significant loss in contractility when compared to native tissue (Figure 2D). The contractile response decreased significantly from 11.9 ± 2.9 to 3.3 ± 1.3 mg/mg tissue at 4 months (3 dogs, $p=0.008$) and 3.6 ± 3.3 mg/mg (4 dogs, $p=0.015$) at 7 months after damage. These functional results are supported by the histological findings showing less muscle content and more connective tissue 3 and 7 months after model generation.

Cystourethrography

Radiograms of the damaged sphincter region showed urethral tissue bulging, indicating loss of function (Figure 3). Urethral tissue dilatation was progressively prominent at the later time points. None of the animals had urethral strictures in the damaged area.

Histology

The damaged sphincter showed a substantial loss of muscle layer with an overall decreased wall thickness (Figure 4). The wall consisted mainly of connective tissues and lamina propria. Anti-desmin confirmed a decrease in muscle content toward the periphery of the sphincter wall at 3 and 7 months postoperatively. There were no signs of inflammation or fibrosis.

Discussion

Our canine model of sphincter insufficiency seems to fulfill the desired criteria mentioned in the introduction. Thus, the damage inflicted was sustained, which should allow long-term studies, sphincter function could be measured using established tools including urodynamic studies, and the model was easily created and maintained.

In our study the dog was selected as the species for sphincter insufficiency for several reasons. Although the lower urinary tracts of sheep, pigs and dogs have tissue characteristics similar to those in humans, tissue changes associated with aging must also be considered [19]. The tissue growth rate in sheep and pigs increases rapidly, which limits the ability to monitor sphincter function over time. In contrast, the tissue growth rate in dogs remains consistent throughout adult life. In addition, dogs have been successfully used for urodynamic evaluations [14-16]. Several anatomical differences exist between the dog and other species, including the horizontal orientation of the body axis, absence of a strong pelvic floor musculature and the floating nature of the bladder. Although nonhuman primates may serve as an ideal model due to their anatomical similarities to humans, this has been questioned [20]. Furthermore, the availability of primates and special housing requirements make this model impractical.

Despite the anatomical differences between the canine model and human, the sphincter muscle functions similarly. The circular orientation of the sphincter muscle (musculus urethralis) in dogs is equivalent to the external urogenital sphincter in humans. Although we demonstrated that damage to the sphincter muscle decreases pressure required for coaptation of the urethra, our model does not replicate the complex pathophysiological mechanism of stress incontinence seen in patients, which usually involves the combination of damage to supporting structures, nerves and muscle. The sphincter insufficiency model we developed generated defined muscle damage, which allowed for accurate assessment of sphincter function. Therefore, this model system may be best used for investigative studies related to the development of bulking agents or cell based therapies for sphincter competency. However, this model may not be suitable for testing pharmacological or hormonal effects on the damaged tissue.

Several methods of sphincter damage in models have been reported previously. The microsurgical approach used in our study to remove sphincter muscle produced rapid and reproducible functional damage to the external sphincter without any evidence of abnormal tissue response, including excessive

inflammation. The surgical techniques we used are simple and reproducible. Myotoxins and electrocautery have been used previously but these methods are known to induce high levels of inflammatory response and do not replicate the clinical conditions in humans [1, 2, 10, 21].

The outcomes of our model generation were evaluated with the standard urodynamic equipment used in patients. Unlike human examinations during which the patient is fully awake, the animals were anesthetized. Because it is well-known that urodynamic results may be influenced by certain anesthetic agents and the depth of anesthesia, we monitored the animals to maintain shallow anesthesia and minimize the effects on muscle relaxation. Using this precaution, all animals had a significant sphincter pressure decrease after surgery. Sphincter function did not return in any of the animals during the 7-month study period. However, the DLP increased slightly after month 3 of observation without any evidence of tissue regeneration in the damaged sphincter region. A possible explanation for this finding may be due to the natural compensation of the adjacent tissue structures. However, the physiological organ bath studies and electrical stimulation of the pudendal nerve confirmed sustained functional decrease of the damaged sphincter region. In addition, the anatomical defects created by sphincter damage remained consistent as demonstrated on cystourethrography, and histological and immunohistochemistry studies. Removal of the sphincter muscle resulted in weakening and bulging of the urethra.

Conclusion and summary.

This study demonstrates that a reliable and reproducible canine model of irreversible sphincter insufficiency can be achieved through microsurgical removal of sphincter muscle tissue. The damaged sphincter muscle resulted in functional loss in all animals. This preclinical model of external sphincter insufficiency may serve as a tool for evaluating clinical applicability of various therapies for the treatment of SUI.

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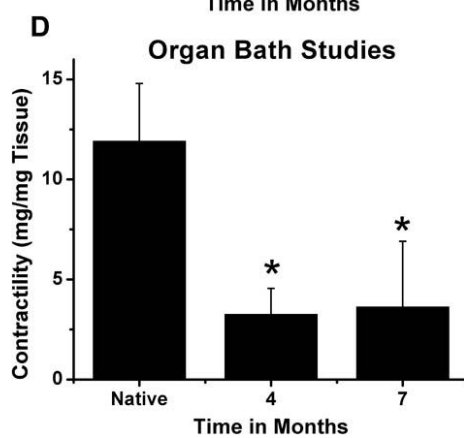
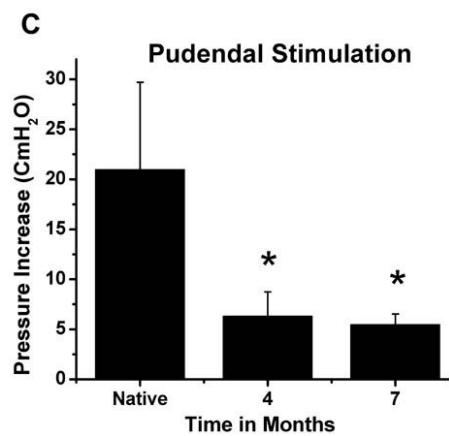
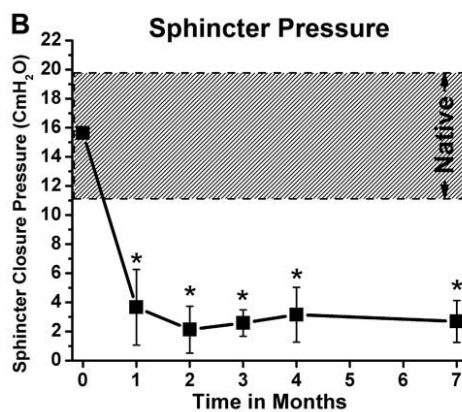
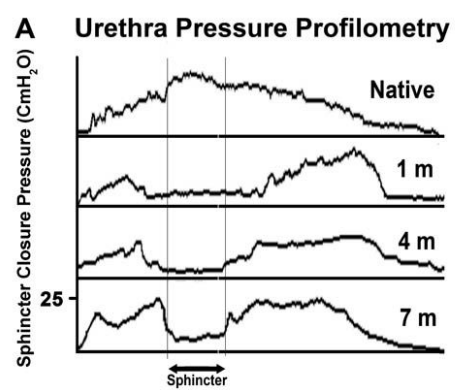
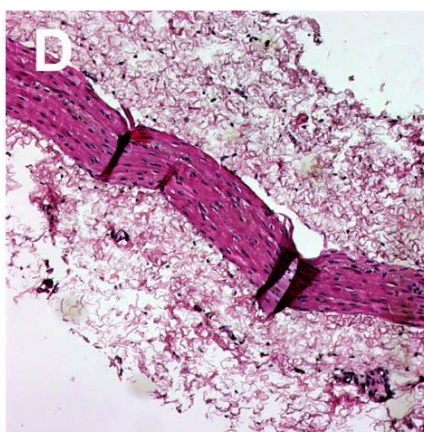
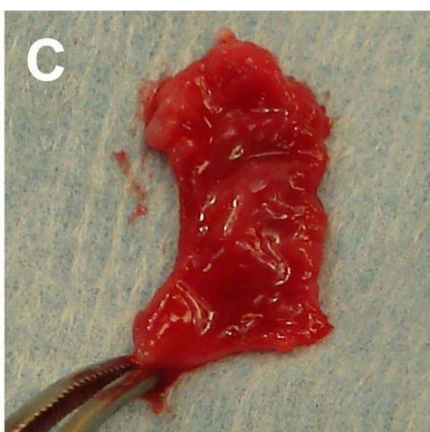
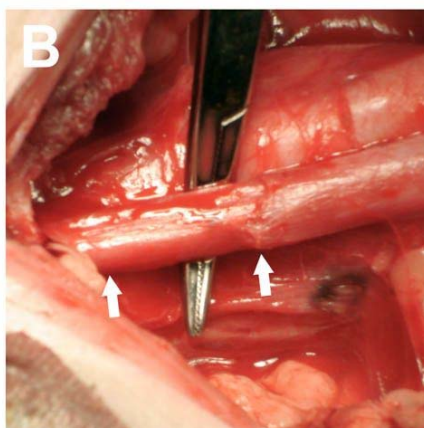
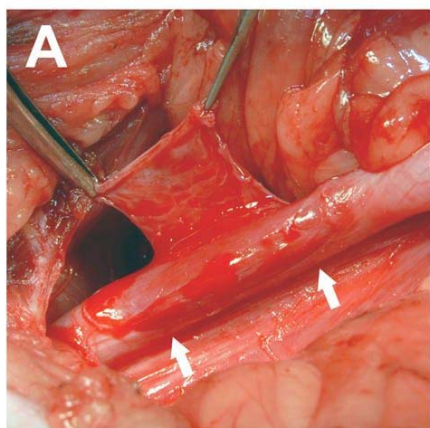
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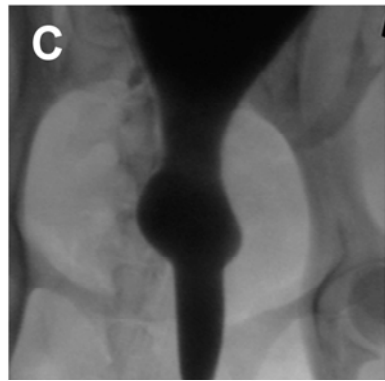
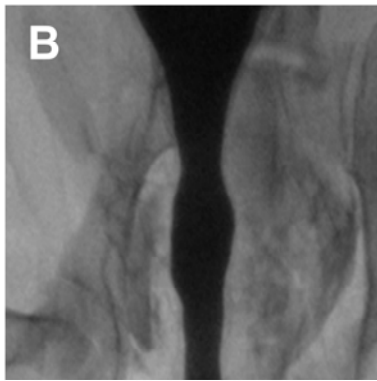
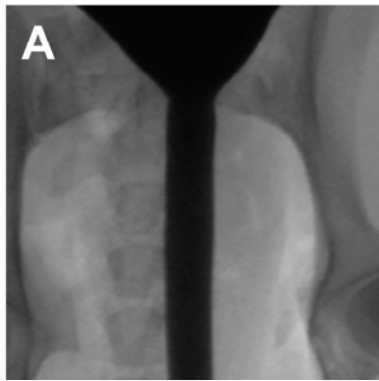
Figure 1. Surgical technique. *A*, sphincter area (arrows 2 cm apart) with half of circumference of sphincter muscle removed. *B*, sphincter muscle after removal of circumferential tissue (arrows 2 cm apart). *C*, removed tissue strip. *D*, muscle layers of removed tissue with the serosa side on top right. *H* & *E*, reduced from 100x

Figure 2. *A*, A representative static UPP at 0, 1, 4 and 7 months after model generation. Pressures in sphincter region decreased after microsurgical removal of sphincter muscle. Animals demonstrated slight compensation with increased pressures proximally and distally with respect to damaged area. *B*, significant decrease occurred in sphincter pressure after removal of sphincter muscle. Pressures remained low for 7-month study period. *C*, sphincter pressure increase during pudendal nerve stimulation decreased significantly after removal of sphincter muscle. *D*, physiological organ bath studies confirm loss of sphincter function with significant lower contractility after removal of sphincter muscle.

Figure 3. Radiography of the bladder neck region shows bulging of urethral wall after model generation. *A*, native sphincter. *B*, 4 months after surgery. *C*, 7 months after surgery.

Figure 4. Histological examination of the sphincter region shows muscle defect after model generation. *A* to *C*, connective tissue and submucosal layer of damaged sphincter region. Note increased diameter of lumen. Scale bar represents 2 mm. *D* to *F*, *H* & *E* stain, reduced from X100. Scale bar represents 100 μ m. *G* to *I*, decrease in muscle content (anti-desmin) of damaged sphincter region. Scale bar represents 100 μ m.





Native

3 Months

7 Months

